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REVIEW

Ecotoxicology of nanomaterials: the role of invertebrate testing

AG Cattaneo[1], R Gornati[1], M Chiriva-Internati[2], G Bernardini[1,3]

[1] *Department of Biotechnology and Molecular Sciences (DBMS), University of Insubria, Varese, Italy*
[2] *Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA*
[3] *Centro Interuniversitario Politecnico di Milano e Università dell'Insubria "The Protein Factory"*

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Abstract

Engineered nanomaterials represent a new and expanding class of chemicals whose environmental hazard is actually poorly determined. The peculiar behavior of nanomaterials makes them much more similar to new chemicals than to the corresponding bulk materials; this feature imposes reliable and standardized evaluation protocols for toxicity and ecotoxicity assessments. General rules for assessing nanotoxicity and the state of the art are periodically published in reports by control agencies. This review highlights the role of invertebrates as valuable and validated test organisms for assessing ecotoxicity of new and/or untested chemicals. The general scarcity of experimental data, their unequal distribution among the different nanomaterials and environmental conditions, the difficulties in manipulating nanomaterials and obtaining stable and homogeneous suspensions, the confusion arising from a not well defined metrics are discussed.

Key Words: engineered nanomaterials; nanotoxicology; invertebrates

Introduction

Assessing the ecotoxicity of previously untested substances, as in the case of nanomaterials, is a challenging task. Therefore, inexpensive, rapid and reproducible methods are preferred, and a coordinated standardization could help in avoiding the waste of resources.

Nanomaterial is a material having at least one dimension 100 nm or less. Nanomaterials can be nanoscale in one dimension (e.g., films), two dimensions (e.g., fibers and tubes), or three dimensions (e.g., particles). Nanoparticles constitute a sub-fraction of what is defined as "colloids" (Christian *et al*., 2008). Chemicals fitting these requirements share protean physical chemistry, which confers very unusual properties to them. The colloid nature, the ability to form aggregates and an appealing potential for practical applications remain nevertheless common features, explaining the growing interest in engineered nanomaterials.

Scientists studying this field were awarded twice with the Nobel Prize for chemistry. This exclusive acknowledgment was firstly given in 1996 for the fullerene synthesis discover (Curl, 1996; Kroto, 1996; Smalley, 1996), and then in 2000 for research on conductive and semi-conductive nanopolymers (Heeger, 2000; MacDiarmid, 2000; Shirakawa, 2000).

Since then, the production of newer engineered nanomaterials and their applications exponentially increased, spanning from cosmetics, drug delivery systems and food additives, to products for waste remediation and fuel and energy production, the so called environmentally friendly nanotechnologies (Tungittiplakorn, 2005; Hollins, 2007). Space and military applications of nanomaterials range at the present from protective clothing, sensors and signals, to propellants and explosives, and more (for reviews see Ruffin, 2004; Glenn, 2005).

At the same time, basic research in the fields of nanoscience and nanotechnology improved rapidly: manufactured and natural nanomaterials branched out as distinctive fields, theories and models developed about their ability to actively interact with environmental and biological systems and the issue of their potential hazard for health and environment has been posed (Oberdörster, 2004; Oberdörster *et al.,* 2005; Moore, 2006; Chun Ke and Qiao, 2007; Oberdörster *et al.,* 2007; Christian *et al*., 2008; Handy *et al*., 2008a, 2008b; Di Gioacchino *et al*., in press).

Nevertheless, nanomaterials remain very poorly tested potential pollutants, in contrast with their

Corresponding author:
Anna Giulia Cattaneo
Department of Biotechnology and Molecular Sciences (DBMS)
University of Insubria
Via J-H Dunant 3, 21100 Varese, Italy
E-mail: annagiulia.cattaneo@uninsubria.it

large diffusion: main difficulties in assessing toxicity are a consequence of their colloidal nature and dynamics, as systems in which smaller or larger aggregates can form in poorly predictable ways, making it difficult to measure shape, size and concentration in the final sample (Service, 2004; Nowack and Bucheli, 2007; Blaser *et al*., 2008; Diegoli *et al*., 2008; Hassellöv *et al*., 2008; Tiede *et al*., 2008, 2009). The ability of nanomaterials to interact with natural soils and porous or colloid substrates allows a long, often unexpected, passive transport to the groundwater (Nowack and Bucheli, 2007; Loux and Savage, 2008; Farré *et al.*, 2009). Important tools lacking in assessing nanotoxicity are sample-related certified standards, reliable measure units and analytical chemical procedures to measure nanomaterials in the environment. Despite a great concern about waste and pollution, the presence of nanoparticles in effluents, sediments or surface waters near urban areas supporting a nanotechnology industry is not yet documented.

The use of approved or certified standards, a basic requirement for good practice in toxicology laboratories, seems far from fulfillment in the case of nanomaterials. In all naturally occurring systems (water, soil, air and their combinations), the organization of the dispersed nanophase depends equally from the physical-chemistry of the manufactured nanomaterials and from that of the environment, as well as from the modalities of suspension. Obtaining a gold standard for every case is far-fetched, redundant and expensive, while the use of a stable internal standard could attain a satisfactory level of laboratory practice. Furthermore, interlaboratory comparisons will improve the characterization and overcome the complexity of nanometrology (Hassellöv *et al*., 2008).

Theoretical prediction of equilibrium represents a pivotal characterization of nanocolloids. Indeed, nanoparticles partially elude the rules of Derjaguin, Landau, Verwey and Overbeek (DVLO) theory requiring remodelling, Nernst equilibrium does not apply, and the charge density at the surface cannot be calculated when the surface area is unknown (Loux and Savage, 2008). The specific surface area exponentially increases as a function of small size, and amplifies the energy of collision between particles due to the Brownian motion, a major event affecting the colloid stability (Casey and Rustad, 2007; Christian *et al*., 2008; Tenne and Seifert, 2009). Experimental evidences, positively relating this parameter to toxicity, endorse speculations about its importance in toxicology (Oberdörster *et al*., 2005; Stoeger *et al.,* 2006).

Another feature characterizing the colloid stability is the zeta potential, i.e. the diffuse surface charge, linked to the chemical nature of the dispersed nanomaterials and to the properties of the continuous phase (pH, dilution, temperature and interatomic distance between others). Its measure gives good information on nanomaterials mobility, aggregation rates and interactions with surfaces: when its value approaches 0 mV massive aggregation occurs (Dunphy Guzman *et al.*, 2006a; Loux and Savage, 2008). Moreover, for magnetically charged particles, the dipole moment is a key feature in their characterization and appears to be related to the toxicity potential (Kumar *et al.,* 2006; Malvindi *et al.,* 2008).

The Critical Coagulation Concentration (CCC) of electrolytes (mol/l) is particularly interesting in evaluating the stability of colloid suspensions in hard and salt water: the stability of suspension is strongly dependent from counterion valence and electrostatic potential at the interface, at least in nearly spherical nanometals (Loux and Savage, 2008).

The recently released notes of the Organization for Economic Co-operation and Development (OECD, 2008; Table 1) include these properties in the endpoints for phase one characterization of manufactured nanomaterials. In addition to them, water solubility and stability of dispersions, crystalline phase, dustiness, crystallite size, TEM picture(s), particle size distribution, surface chemistry, photocatalytic activity, pour density, porosity, octanol-water partition coefficient, redox potential, and radical formation potential are listed.

The second tool, i.e., a reliable measuring unit expressing toxicity, gave matter of discussion. Particle size, firstly suggested as a highly appropriate unit of measure related to toxicity, declined in popularity in recent years: the size of aggregate, not particles, seems to be more informative (Pauluhn, 2009). An alternative method, expressing nanomaterials toxicity based on mass or on surface area, seemed to be satisfactory (Oberdörster *et al*., 2005; Stoeger *et al.,* 2006). However, a revision of published data in an attempt to explain non-linear dose-response toxicity of some nanomaterials revealed that surface-to-mass area seemed to be preferred to surface-to-size, and the number of particles performed as well (Wittmaack, 2006). A lively discussion followed with every scientist supporting his or her own reasons (Oberdörster *et al*., 2007; Stoeger *et al.,* 2007; Wittmaack, 2007). The problem, as claimed in many works, is probably more complex, and additional information is needed to obtain optimally informative metrics (Kandlikar *et al*., 2007; Teeguarden *et al*., 2007; Gornati *et al.,* in press). Nevertheless, the number-based metrics proposed by Wittmaack (2006) adds predictive value to the existing experimental data on nanomaterial ecotoxicity, which remain mainly, if not exclusively, expressed as concentrations (ppm, molarity or w/v). This method is simple, generally accepted and comparable with the conventional units for corresponding bulk chemicals and should be used at least until adequate standard metrics and samples are available.

Another regrettable lacking matter is the systematic measure of elements released by dissolution. Dissolution is dependent from the chemical nature and size of the nanoparticle, as well as from environmental variables, such as pH and temperature (Meulenkamp, 1998; Vogelsberger, 2003; Hardman, 2006). Consequently, the measured effect and the eventually observed toxicity could be not a feature of the nanomaterial itself, but of its degrading products. Dissolution has been very rarely documented, while these kinds of events are well known and frequently postulated

Table 1 Guidelines for assessing toxicological risk of nanomaterials. National and supranational agencies

AGENCY	COUNTRY	DOCUMENT ID.	YEAR
CST	UK	Nanosciences and Nanotechnologies: A Review of Government's Progress on its Policy Commitments (http://www2.cst.gov.uk/cst/business/files/nano_review.pdf)	2007
DEFRA	UK	Nanotechnologies - Research reports (http://www.defra.gov.uk/environment/nanotech/research/reports/index.htm)	2009
RCEP	UK	Novel Materials in the Environment: The case of nanotechnology (http://www.rcep.org.uk/reports/27-novel%20materials/documents/Novel-Materials-report.pdf)	2008
European Commission	EU	European activities in the field of ethical, legal and social aspects (ELSA) and governance of nanotechnology. (http://cordis.europa.eu/nanotechnology/)	2008
OECD		Series on the safety of manufactured nanomaterials – Nr. 6 (http://www.olis.oecd.org/olis/2008doc.nsf/LinkTo/NT000034C6/$FILE/JT03248749.PDF)	2008
OECD		Series on the safety of manufactured nanomaterials – Nr. 8 (http://www.olis.oecd.org/olis/2009doc.nsf/LinkTo/NT000029E6/$FILE/JT03263204.PDF)	2009a
OECD		Nanotechnology research resources by country (http://www.oecd.org/countrylist/0,3349,en_21571361_41212117_42325621_1_1_1_1,00.html)	2009b
OECD		Guidelines for the testing of chemicals bioaccumulation in terrestrial oligochaetes (http://www.oecd.org/dataoecd/11/8/42551309.pdf)	2009c
ISO, IEC, NIST and OECD		International workshop on documentary standards for measurement and characterization for nanotechnologies (http://www.standardsinfo.net/info/livelink/fetch/2000/148478/7746082/index.html)	2008
US EPA	USA	Nanoscale materials: Stewardship program. Interim Report (http://www.oecd.org/dataoecd/33/55/42061387.pdf)	2009
SCENIHR	EU	Risk Assessment of Products of Nanotechnologies (http://nanotech.lawbc.com/tags/scenihr/)	2009

CST: The Council for Science and Technologies; DEFRA: The Department for Environment, Food and Rural Affairs; US EPA: Environmental Protection Agency, USA; SCENHIR: Scientific Committee on Emerging and Newly Identified Health Risks; UE: United Europe; OECD: Organisation for Economic Co-operation and Development; ISO: International Organization for Standardization; IEC: International Electrotechnical Commission; NIST: National Institute of Standards and Technology; RCEP: Royal Commission on Environmental Pollution

(Lovern and Klaper, 2006; Biju *et al.,* 2008; Handy *et al.,* 2008b; Heinlaan *et al.,* 2008).

The common efforts of the worldwide control agencies and governmental organisms finally achieved their goal in coordinating the resources, and periodically updated guidelines for risk assessment of nanomaterials have been published (Table 1). The policy applied by control agencies to expert recruitment, research funding and targeting the point of interest have been critically reviewed over time (Oberdörster *et al.,* 2005; Dunphy Guzman *et al.*, 2006b; Rickerby and Morrison, 2007; Paradise *et al.*, 2008; Wilhelmi, 2008).

The US EPA in its recently released Nanoscale Materials Stewardship Program (NMSP), elaborated an assessment of knowledge about nanomaterials: data scheduled in databases (Nanowerk Nanomaterials Database and Wilson Center Project on Emerging Nanotechnologies (PEN) Inventory of Nanomaterials in Consumer Products), or directly submitted to the NMSP were collected and compared. Sharing a part of commercially available products from those for research use only or under development, the engineering process standardization and description was the best characterized (EPA, 2009; Table 1). An overall picture of the statistical elaboration of the data set evidenced that morphology, physical chemistry and production processes have been characterized by 50-90 % in all nanomaterials. This means that validation of producing processes, availability of approved standard for pristine materials and approved nomenclature are near to be reached purposes for the largest part of known engineered nanomaterials. The following best characterized point was the knowledge about professional risk factors in handling and manipulating nanomaterials. The risk was assessed for 50 % of known materials. Rapid improvement in this field should be expected from the protocol planned for the period 2003-2013 and involving risk insurance companies (Lauterwasser, 2003; OECD, 2009a; Table 1).

Experimental data about acute and chronic ecotoxicity and environmental fate score lower by far: tested materials do not exceed 20 % in this respect. Any effort to bridge the gap with an adequate set of measures in this field should be encouraged in the near future.

While natural systems represent the real target of these investigations, artificial laboratory conditions can be preferred for practical reasons. First, the steps of knowledge can speed up by using a combination of test media mimicking the natural environment, and test species adapted to the laboratory, representative of the field. Acute lethality should be conveniently studied first, long-term toxicity tests following as needed, focused on growth and reproduction as well as morphology and behavioural changes. Simplified or natural food webs should be tested to identify the levels and the risks for bioaccumulation and biomagnification (Crane *et al.,* 2008), and modelling of exposure has been proposed (Mueller and Nowack, 2008).

Endpoints for phase one assessment of environmental toxicity of nanomaterials comprise a list of short and long term effects on species inhabiting pelagic, sediment/benthic, soil and other terrestrial habitats (Gourmelon and Ahtiainen, 2007).

Invertebrates as test organisms for environmental pollution studies

The invertebrates represent valuable organisms for environmental pollution studies. In addition to be among the most widely distributed living organisms on the Earth and offer the opportunity to explore nearly every ecological niche, they have a relatively short life span, reproduce quickly at higher rates and are sensitive to pollutants. Invertebrate-based tests are cost-effective, reasonably quick and easy to perform with reproducible standard protocols for multicentered trials. These organisms are indeed particularly convenient as test species to pioneering ecotoxicity studies on newer chemicals, and nanoparticles among others (Gourmelon and Ahtiainen, 2007). Other physiological features of some invertebrates are amictic reproduction, warranting a large number of identical clones, and production of resting eggs, exploited to produce commercial test kits. Wild specimens generally adapt quickly to laboratory conditions, making standardization easily obtainable.

Additional advantages are the highly reproducible staging for larval progression, the small size of adult individuals with transparent bodies, and a stereotyped behavior with easily recognizable disruption. These features facilitate the collection of valuable statistical data at the three levels important for species threatening: survival (quantified by LC_{50} or Median Lethal Concentration), growth rate, and fertility (expressed as latency and length of reproductive life, number of offspring per life cycle, and offspring viability rates). Chronic studies provide information on sub lethal effects, such as active swimming, feeding and avoiding predation capabilities: NOEC (Non Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) are the mostly used in this type of approach. Besides, an array of changes at cellular and sub cellular level can be followed in invertebrates to inform on the mode of actions of chemicals. Despite their wide use in other fields, environmental and ecotoxicological genomics, proteomics and metabolomics (Snape *et al.,* 2004) in invertebrates are at the very beginning. The increasing interests in these areas raise the hope for a rapid enhancement in the near future, with useful applications to nanoparticle studies (Snape *et al.*, 2004; Hines *et al.*, 2007; Iguchi *et al.*, 2007; Poynton *et al.*, 2007; Soetaert *et al.*, 2007; Ralston-Hooper *el al.*, 2008; Steinberg *et al.*, 2008).

Differently from prokaryotes (Fang *et al.*, 2007; Heinlaan *et al*., 2008; Holbrook *et al.*, 2008), invertebrates are able to intake nanomaterials dispersed in the environment by different ways: direct ingestion or from contaminated preys, water filtration, inhalation, and surface contact. Some degrees of biomodification occur, at least in daphnids (Oberdörster *et al.*, 2006a; Roberts *et al.*, 2007; Baun *et al.*, 2008; Filella *et al.*, 2008), and compartmentalization of nanosized contaminants in selected tissues and intracellular organelles has been documented (Moore *et al.*, 1997; Leeuw *et al.*, 2007; Tortiglione *et al.*, 2007; Ingle *et al.*, 2008; Koehler *et al.*, 2008; Oughton *et al.*, 2008; Tedesco *et al.*, 2008).

Invertebrates largely enter the food chain mainly at intermediate levels, and represent a powerful vehicle for recycling pollutants deposited in the sediments. As predator of bacteria, plants, algae and other invertebrates, or feeding on substrates, they become the preferential prey of larger organisms: humans, fish and birds, which, in turn, represent a great deal of the human diet. The risk for humans is possibly related to the doses, therefore, bioconcentration, bioaccumulation and biomagnification raise a matter of concern. Their rates, clearance time and fate of the contaminants through the food chain should be measured whenever possible (Allen *et al*., 2005; Rocha *et al*., 2005; Emerich and Thanos, 2006).

Studies on ecotoxicological risk assessment for nanomaterials should follow rules and protocols accepted for previously untested chemicals; whenever possible comparison with comparable bulk materials should be considered. The weak stability of nanocolloids represents a problem to be solved: a comprehensive and recent paper in this particular issue reports the generally accepted approaching scheme (Crane *et al.*, 2008). Briefly, a tired protocol evaluates first acute lethality tests of contaminated waters and sediments on simple organisms: bacteria, monocellular algae and invertebrates. Long term tests should follow, if convenient, and data should be predictive of lethality, growth, reproduction and offspring viability. Organisms of greater complexity (from bacteria to algae, invertebrates and eventually fish and birds) will be included as needed by the main aim of the study. The preference should be given to standardized test and to test species ecologically representative and well adapted to standard laboratory conditions: the control agencies for environmental contaminant surveillance schedule tests on daphnids, in addition to bacteria, algae and

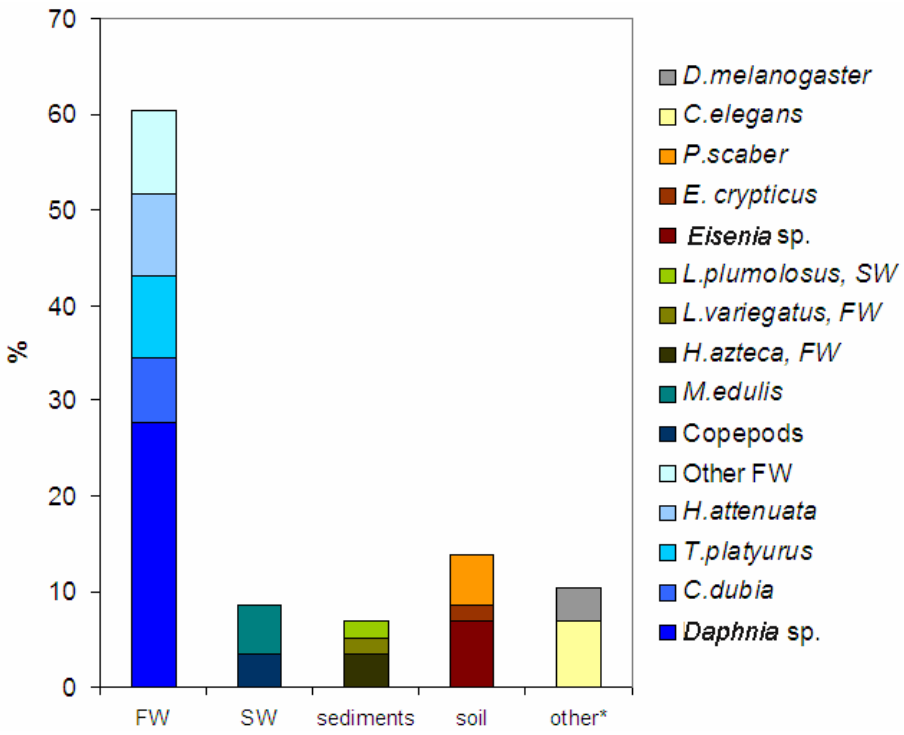

Fig. 1 Percentage of nanotoxicological studies performed in different test media and species. FW: freshwater; SW: salt (marine or artificial sea) water. Other*: liquid media (*C. elegans*), foods (*D. melanogaster*)

fish, as key step for assessing aquatic toxicity. Other invertebrates are recommended as standard test organisms to test benthic waters, sediments, soil, ingestion or skin contact, or for artificial food web analysis (Crane *et al.*, 2008). Once the material under investigation has shown aquatic toxicity and a trend to accumulate in soils and sediments, tests on these substrates should follow. Finally, a set of experiments on organisms playing a higher role in the alimentary chain should be appropriate, in the case of suspected or documented bioaccumulation or biomagnification interesting the food chain.

Invertebrates used in toxicity studies on nanomaterials: main characters and habitats

This section will present the features which characterizes the species of invertebrates which underwent nanotoxicity assessment up until today and the rational for their selection. As shown in Fig. 1, species entering nanotoxicity studies are few and unevenly distributed in different environmental samples. Test on the water column are prevalent, mainly conducted on daphnids and other cladocerans, while studies on sediments are neglected, despite the emphasized trend of aqueous nanocolloids to be unstable and precipitate. Again, in some cases practical reasons (kit availability, lab expertise, others) seems to prevail on recommended protocols in determining the choice of test species. The main taxonomic classification of invertebrates considered in the studies on nanotoxicity is presented in Table 2, together with codes of standard tests validated by at least one of the control Agencies listed in the legend of Table 1.

Testing the water column: pelagic and littoral organisms

Tests on water column or water-suspended sediments should be performed in standardized media. Commercial mixtures, like those used for aquaria or provided in kits, should be preferred to comparable solutions obtained at the lab bench by mixing high-grade chemicals in demineralized water. These solutions, in fact, are expensive, poorly reproducible and at higher risk of leaving unsafe concentrations of heavy metals in the test medium (ECETOC, 2003).

Control agencies recognize Daphnids (*Daphnia magna, Daphnia pulex* and *Ceriodaphnia dubia*) as "first choice" test organisms for validated ecotoxicological tests (Joncxyk and Gilron, 2005). The Daphnia Genomics Consortium (DCG, http://daphnia.cgb.indiana.edu) promotes sequencing projects of the entire genome of *Daphnia* sp. as model organisms for ecology. These small crustaceans, widely distributed in all aquatic habitats with the only exception for extreme environments, show all the features mentioned before as highly convenient for testing. If maintained in optimal conditions (pH = 7.2-8.5; t = 20 °C; hard water=160-180 mg/l or 80-90 mg/l $CaCO_3$, related to species), Daphnids adapt quickly in the laboratory. Stereotype behaviors, which involve filtration feeding, jerky motion in swimming and anti-photo tactic movements, are in part genetically determined and appear to be sensitive to chemical pollution.

Table 2 List of Invertebrate species used in testing nanoparticles toxicity

SPECIES	CLASS	ORDER	CODES OF VALIDATED TESTS	APPROVED BY ASTM/EPA/OECD/EU/ISO
FRESHWATER				
Crustaceans: *Daphnia magna* and *D.pulex, Caeriodaphnia dubia Chydorus sphaericus**	Branchiopoda	Diplostraca	Acute: EPA850.1010; EPA821-R02.013, OECD202, ASTME-12095-01. Sublethal: EPA850.1300; OECD211, ASTME1193-97, ASTME-12095-01	All (only Daphnids)
Crustaceans: *Thamnocephalus platyurus**	Branchiopoda	Anostraca		None
Rotifera: *Brachionus calyciflorus*	Monogononta	Ploimida	Acute: ASTME-1440-91 Sublethal: ASTME-2317-04	ASTM/EPA
Cnidaria: *Hydra attenuata*	Hydrozoa	Hydroida	ASTM: STP921-EB	ASTM/EPA
Molluscs: *Elliptio complanata**	Bivalvia	Unionoidea		None
SALT (ESTUARINE, SEA WATER)				
Crustaceans: harpacticoida copepods	Maxillopoda	Harpacticoida	ASTME-2317-04, OECD 254	ASTM/EPA/OECD
Molluscs: *Mytilus edulis*	Bivalvia	Mytiloidea	ASTME-2122-02, EPA850.1050	ASTM/EPA/OECD/EU
FRESHWATER SEDIMENTS				
Crustaceans: *Hyalella azteca*	Malacostraca	Amphipoda	ASTME-1706-00, OECD 251, EPA850.1735, EPA600/R99.064	ASTM/EPA/OECD
Worms: *Lumbriculus variegatus*	Oligochaeta	Lumbriculida	ASTME1688-00, EPA 823-f-00-002; OECD2007: new proposal	ASTM/EPA/OECD
SEA WATER SEDIMENTS				
Crustaceans: *Leptocheirus plumulosus*	Malacostraca	Amphipoda	ASTME1367-99, EPA850.1735; EPA 600/R01/020, OECD 252	ASTM/EPA/OECD
SOIL				
Earthworms: *Eisenia* sp.	Oligochaeta	Haplotaxida	ASTME1676-04 (toxicity and bioaccumulation); EPA850.6200; OECD207/211 (acute/chronic)	All
Potworms: *Enchytraeus crypticus*	Oligochaeta	Enchytraeidae	ASTME1676-04 (toxicity and bioaccumulation); ISO 16387:2004; OECD 207/FKZ: 204 67 458	ASTM/ISO/OECD
Crustaceans: *Porcellio scaber**	Isopoda	Oniscidea		None
OTHER				
Nematodes: *Caenorhabditis elegans**	Chromadorea	Rhabditida		Model organism
Arthropods: *Drosophila melanogaster**	Insecta	Diptera		Model organism

The tests approved by control organisms and test codes, whenever available, are reported (see even Burton *et al.*, 2003; Crane *et al.,* 2008). Asterisks: not yet validated by control agencies.

Short life cycles and amictic reproduction are the rule: deposition of non-resting eggs in the dorsal brood chamber (from which juvenile female are released) gives rise to large, rapidly evolving clones. A great level of body transparency permits non-invasive exploration of brood chamber and of gut content. Sexual cycle follows non favorable changes in natural habitats (freezing, drought and others): 1-2 resting eggs are deposited in the ephyppium, a detachable modification of the carapace, and male individuals are produced, whose sperm is haploid, tailless but motile. Sexual cycles are more frequent in *C. dubia* (Ebert, 2005). Commercial kits, based on standardized acute and chronic toxicity tests on Daphnids, contain "cysts" (ephyppia) and use hard freshwater as the test medium (Persoone *et al.*, 1994; Centeno *et al*., 1995; Ruck, 1998; Lazorchak *et al.*, 2008).

Testing the water column: pelagic (or littoral)/ benthic organisms

Chydorus sphaericus is a Daphnid-like cladoceran whose small spherical body is transparent. The dorsal brood chamber is present only in adult females, and reproduction is mainly amictic. Feeding and swimming patterns are similar to those described for daphnids. Its ability to change habitat from pelagic to bentic confers to him the property of an useful alternative to daphnid testing, used since 1991 (Havens, 1991). While not yet approved by control agencies a test for acute toxicity on *C. sphaericus* has been recently proposed (Pieters *et al.*, 2008; Velzeboer *et al.,* 2008).

Copepods are other organisms in this group, perhaps the dominant ones, at least for the very large number of species and the ability to colonize a wide range of aquatic ecosystems. Studies on nanomaterials have been performed only in marine species, by far the most numerous species. Different from Daphnids, their body is segmented and poorly transparent. Pelagic larvae (nauplia) develop slowly (within months) into sexually (male and female) differentiated adults. The life span is comparably long and variable between species. No resting eggs are produced, but in unfavorable environmental conditions adults enter diapause. Benthic habits are restricted to the adult life. Swimming and crawling-swimming movements on substrates are powerful (90 m/h). Copepods feed on suspended particles (like algae, bacteria and organic matter), and are validated test organisms for ecotoxicology (Anderson *et al.*, 2001).

Thamnocephalus platyurus is a big freshwater crustacean, with a segmented and poorly transparent body. While two distinct sexes have been described (Byron and Ponder, 1949), resting eggs can be produced. A commercial kit supplies this material to perform acute studies. However, the test time (24 h for hatching plus 1 h for *in vitro* exposure to contaminants) is too short to allow a full maturation of the larvae (Williams, 2007). The test is widely used, but not included in standard validated methods (Centeno *et al.,*1995).

Other benthic organisms are useful for long-term toxicity studies, such as the cnidarian *Hydra attenuata*, a freshwater, relatively small organism. Lacking a mesoderm hydras belong to the group of diploblastic animals; their tubular body develops a head, terminated by an oral apparatus with a crown of tentacles, and a basal disc (adherent to the substrate) at opposite extremities. Reproduction proceeds by clones; they are generated by budding from the distal third of their body, near the basal disc. A gradient of activators regulates head formation and regeneration by morphallaxis, and inhibits budding in younger individuals. Differentiation into true hermaphrodite and sexual reproduction are induced by unfavorable conditions. Toxicity tests have been described: clubbing movements of tentacles are early signs of exposure to toxic agents, before reproductive changes or death (Davies and Freeman, 1995; Blaise and Kusui, 1997; Holdway, 2005). Hydras are interesting models for aging: no tissue aging processes or age-related enhanced mortality have been observed in hydras over a period of 4 years. However, some degeneration follows the induction of sexual reproduction, at least in some species, but not in *H. attenuata* (Martinez, 1998; Austad, 2009).

Chronic toxicity was assessed on the bivalves, *Mytilus edulis* (blue mussel) and *Elliptio complanata*, two species feeding while filtering large amounts of sea and freshwater, respectively. The model was restricted to quite large, long living and mainly sessile organisms. The sea species is long 3-7 cm, and inhabits the intertidal, infra and circa-littoral zones. *E. complanata*, whose habitat is restricted to freshwater, can reach 25 cm in length and 10 years of age. Both species reproduce sexually. Their importance as test organisms for ecotoxicology is linked to the role played in the food chain.

The Rotifer *Brachionus calyciflorus* was studied under exposure to nanomaterials as a component of a simplified food web: algae and bacteria were predated by a protozoan, which in turn was the rotifer prey; both fed fish (Holbrook *et al.,* 2008). Testing artificial food web containing rotifers is a validated procedure (Snell, 2005). The acute test has been validated, and a commercial kit for acute (24 h) and short-chronic (48 h) toxicity tests is available: the kit supplies resting eggs and the test is performed on newly hatched larvae (Persoone *et al*., 1993).

Brachionidae are eutelic, small loricate organisms, with one-pieced, thin and transparent lorica, a small corona (the oral apparatus), a single gonad, and a long "foot" used for anchoring. Both free swimming and sessile forms are present. Amictic and sexual reproductive cycles can alternate in relation to environmental conditions. In favorable conditions, non-resting eggs externally attached to the root of the maternal foot hatch within 12 h. Immediately before periods of freezing or drying, small defective males lacking a digestive system appears. Insemination of females leads to deposition of resting eggs, from which a new generation of diploid females hatches when environmental conditions improve. The life span is short (ca. 2-3 weeks), but the total number of offspring is large.

Testing sediments: fresh-water and marine benthic organisms

These animals inhabit the deepest zone of water bodies and are useful to study sediments, on which they are feeding. Three species have been tested in nanoecotoxicology, all representing validated test organisms.

Hyalella azteca (Family: Hyalellidae) and *Leptocheirus plumulosus* (Family: Aoridae) are small shrimps inhabiting freshwater and estuarine sediments respectively, and feed on sediments or suspended particles. Sexual reproduction is the rule, with two dimorphic sexes (larger males). Tests for acute and chronic toxicity are performed in younger individuals and the experimental time should not exceed 4 weeks for *H. azteca* and 10 days for *L. plumulosus* (Borgmann *et al.*, 2005).

The worm *Lumbriculus variegatus* (Annelida, Oligochaeta, Lumbriculidae) inhabits freshwater sediments in ponds, lakes and marshes, where it feeds on decaying vegetation and microrganisms. It can be occasionally found in silty sediments in deeper waters. Sexual reproduction begins between true hermaphrodites, with formation of transparent cocoons containing 4-11 fertilized eggs. Small worms hatch in 2 weeks without a previous larval stage. In laboratory conditions, the worms are smaller (4-6 cm), never reach sexual maturity and reproduce by spontaneous asexual fragmentation followed by rapid regeneration of a complete worm from surviving fragments. Regeneration is also described in natural conditions, fragmentation is a rapid and common response to injuries, like body compression. Its use as a test organism in ecotoxicology is validated (EPA 823-f-00-002).

Testing soils: terrestrial organisms

The earthworms *Eisenia* sp. (*Eisenia foetida* and *Eisenia veneta,* Annelida, Oligochaeta, Lumbricidae) and the potworm *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae) strictly depend on soil, as their habitat and food source, are easy available, growth rapidly and enter the food chain through fish and higher Vertebrates. Earthworms and potworms have a segmented body with cephalo-caudal symmetry. The cephalic portion ends in a clitellum, characterizing sexually mature individuals. Toxicity tests, however, require younger individuals. Both groups are true hermaphrodite, each individual developing a complete male and female set of reproductive organs, with two pairs of testes and one pair of ovaries. While able to completely regenerate from fragments if injured, sexual reproduction is the rule (Dominguez *et al.*, 2003). *Eisenia* sp. and Enchytraeidae are validated test organisms for soil ecotoxicological studies (EPA850.6200; OECD207/211; ISO 16387:2004; Egeler *et al.*, 2009).

The isopod *Porcellio scaber* is another terrestrial organism. It is heavily pigmented and has a ventral brood pouch which characterizes sexually mature females. It hosts fertilized eggs (by sexual reproduction with internal copulation) and hatched larvae as only young complete individuals are released. This crustacean is common in the wooded soil; its diet is mostly on vegetal debris. Life span (up to 3 years) and reproduction rates are sensitive to a number of environmental changes and pollutants.

The OECD recently released updated guidelines for testing the bioaccumulation of chemicals in soils, using earth- and pot-worms as test organisms (OECD 2009c). *P. scaber*, instead, is a test species not yet validated; however, it is the only terrestrial crustacean useful for soil pollution testing.

Model organisms: Caenorhabditis elegans and Drosophila melanogaster

Studies on the toxicity of nanomaterials have been performed on the larvae of two species considered to be model organisms for developmental and cell biology and genetics: the nematode *C. elegans* and the fruit fly *D. melanogaster*.

D. melanogaster is perhaps the prototype of model animal organisms and its genome has been completely sequenced (Adams *et al.*, 2000). It is a small fly, with sexual dimorphism and sexual reproduction. Eggs mature outside the female body; larvae development is by stages. The soil nematode *C. elegans* is easily cultured in laboratory conditions on agar plates. Viable worms can even be stored in frozen stocks indefinitely. The worm body is tiny (about 1 cm long) and transparent, eutelic (100 cells ca) and moving with swimming motion. Two sexes are described: a self-fertilizing true hermaphrodite, with two intra-abdominal gonads, one producing sperm, the other eggs. It is able to give rise to about 300 larvae in its life course. Males are occasionally and rarely produced ($1/10^3$). They are slightly smaller than hermaphrodites, from which they differ for the presence of the only male gonad in the abdomen and the shape of the tail, carrying the organ for the internal copulation, not always followed by fertilization. Sex is genetically determined (hermaphrodites are XX, males X0), however environmental or dietary changes can convert hermaphrodites born from mating into males, but it never occurs with amictically produced hermaphrodites (Hart, 2006; The *C. elegans* sequencing Consortium, 1998).

Toxicity of engineered nanomaterials to Invertebrates

While natural and engineered nanomaterials can overlap and share common properties, this review has been limited to manufactured nanomaterials and to the related ecotoxicological risks.

Nanotoxicology studies, in which the invertebrate plays a significant while intermediate role, remain poorly and unevenly distributed, in spite of great attention reserved to nanotoxicity in humans, cultured cell lines and vertebrate organisms (Chun Ke and Qiao, 2007; Duffin *et al*., 2007; Farrè *et al*., 2009; Shvedova *et al*., 2009; Di Gioacchino *et al.*, in press; Gornati *et al.*, in press).

Environmental nanotoxicology remains almost confined to the aquatic environments, mainly freshwater, while studies on contaminated sediments and non aquatic environments are scarce, accounting altogether for 30 % of the total,

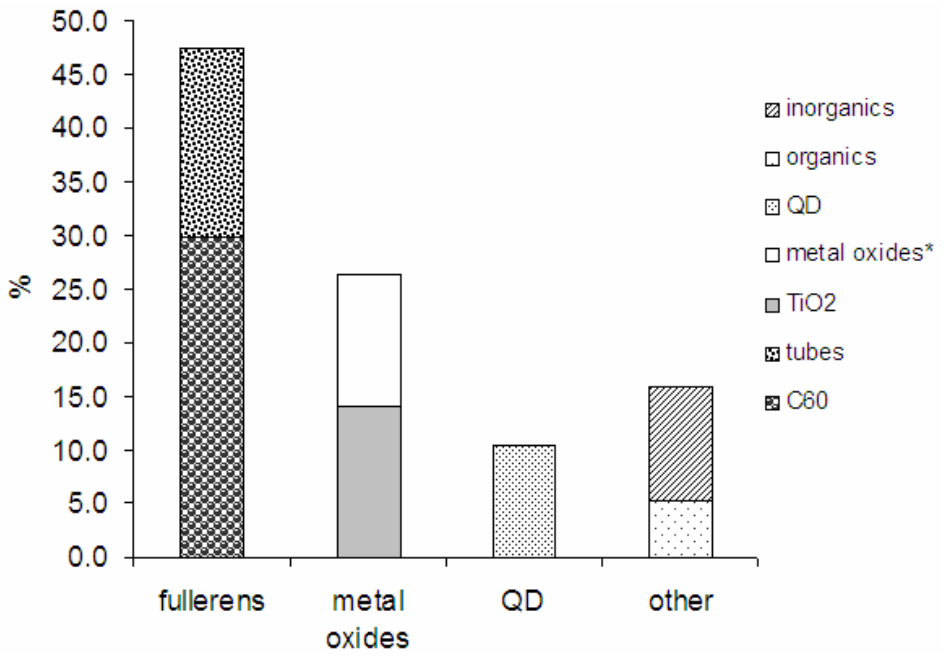

Fig. 2 Percent of ecotoxicological studies on different nanomaterials. Fullerene: C_{60} and carbon nanotubes (CNT); metal oxides: TiO_2 and metal oxides plus TiO_2 plus (*), QDs: quantum dots; others: organic (sucrose polyester oil, polymethyl-methacrylate, NIPAM/BAM) and inorganic (upconverting phosphors, glass wool, nanometals) nanomaterials not included in the chemicals classes mentioned before.

including observations on model organisms maintained in artificial aqueous medium (*C. elegans* and *D. melanogaster*). The most studied test species by far are daphnids (*D. magna, D. pulex* and *C. dubia*) (Fig. 1). The most studied materials are fullerenes and metal oxides that altogether represent 70 % of the available literature in this field (Fig. 2).

Fullerenes

Carbon nanomaterials captured great attention for both historical reasons and convenience. Fullerenes are organized as nearly spherical nanoparticles, the so called buckminsterfullerenes (nC_{60}, nC_{70}, and fullerols, their hydroxylated derivatives) or as single, double or multi-walled carbon nanotubes (SWCNT, DWNCT and MWCNT, respectively). These materials can be toxic for higher organisms: they are able to cross the membrane of eukaryote cells, accumulating in lysosomes and mitochondria, as well the blood-brain barrier, reaching the olfactory bulb in fish and mammals via the olfactory nerve (Oberdörster, 2004; Oberdörster *et al*. 2005; Tin-Tin-Win *et al.* 2008). Moreover, in human cell cultures, SWCNT determine changes in the expression of several genes, mainly involved in apoptosis (Cui, *et al*., 2005; Sarkar *et al*., 2007).

Fullerenes have a very wide application range, from cosmetics to computer engines, and tons are produced yearly. Their waste in the environment is significant and safety is poorly granted by their hydrophobicity, which partially prevents miscibility with superficial water (Heymann *et al.*, 1996; Deguchi *et al.*, 2001; Fortner *et al.*, 2005). While protective effects of fullerenes against other more powerful contaminants were sporadically described (Baun *et al.,* 2008), the potential risk of heavy pollution with fullerene poses major environmental concern and need for studies on adequate models, invertebrates among others. The toxicity of fullerene in invertebrates (Table 3) is variable and related not only to nanoparticle properties, but even to the methods adopted for suspension in water and to the test species.

The link between lethality rates and methods for obtaining suspension was determined for nC_{60} in *D. magna*, perhaps the most sensitive species. Suspensions prepared by long stirring alone seemed to be safe: the $C_{max} \cong 35$ ppm did not reach the LC_{50} even when the test time was prolonged to 96 h. Stirring plus sonication reached an intermediate toxicity, while the solution retaining some traces of THF, added as solvent, was the most toxic. This toxicity apparently is not linked to the residual solvent as, in the absence of fullerene, traces of THF were not toxic (Lovern and Kapler, 2006; Oberdörster *et al.*, 2006a; Zhu *et al.*, 2006; Lovern *et al.*, 2007; Spohn *et al.*, 2009; Zhu *et al.*, 2009). LC_{50} was higher for *D. pulex* while the test time in this case was shorter (Klaper *et al.*, 2009). Other species like *T. platyurus*, *H. azteca* and harpacticoida copepods, seemed to be more resistant and did not show acute toxicity (Oberdörster *et al.*, 2006a; Blaise *et al.*, 2008). Fullerenes added to soils seemed to be ineffective on survival rates of earth- and pot-worms (Baird 2007; Scott-Fordsmand *et al*., 2008).

D. magna appeared to be the most sensitive species even to sublethal effects. Exposure to nC_{60}

Table 3 Summary of lethality data for fullerenes

Fullerene	Species	LC_{50}	Time	Notes
nC_{60} in THF	*D. pulex*	0.5-5.0 ($LC_{30/75}$)	24 h	
nC_{60} stirred/sonicated	*D. magna*	0.8, 0.46 ppm	48-96 h	
		7.9, 10.51 ppm		
nC_{60} stirred		>35 ppm		
	D. pulex	LC_{40} = 100 ppm	24 h	
	harpacticoida copepods	Never reached	48-96 h	No mortality effects
	H. azteca	Never reached	96 h	
	T. platyurus	Never reached	24 h	
nC_{60} added to soil	*E. veneta*	Never reached		No mortality effects
	E. crypticus	Never reached		No mortality effects
$nC_{60/70}$	*D.pulex*	Never reached	24 h	No mortality effects
fullerols	*D.pulex*	LC_{40} = 100 ppm	24 h	
SWCNT	*D.magna*	2.42 ppm	48 h	
	T. platyurus	Never reached	24 h	No mortality effects
	H.attenuata		96 h	
	A. tenuiremis		35 days	
	L.variegatus		28 days	
	D.melanogaster			
DWCNT	*E. veneta*	Never reached	28 days	No mortality effects
MWCNT	*D .magna*	22.75 ppm	48 h	
	C. dubia	7 % survival at 40 ppm	48 h	
	H. azteca	>264 ppm	10 days	
	L. plumulosus	68 ppm		
	L.variegatus	Never reached	28 days	No mortality effects

LC_{50}: Median Lethal Concentration; CNT: Carbon NanoTubes: SW: Single Walled, DW: Double Walled, MW: Multi Walled.

(10 ppm, 48 h) increased the heart rate and amplified (and partially disrupted) stereotypical movements in swimming and feeding. Fullerols had only fable effects, reversible within 30 min (Lovern and Klaper, 2006; Lovern *et al.*, 2007). Long-term (21 days) exposure to stirred nC_{60} solutions reduced reproductive rates. Moreover, the final population was significantly reduced, despite some degree of adaptation (NOEC = 1.0 ppm, LOEC = 2.5 ppm; Oberdörster *et al.*, 2006a). Oxidative stress was observed in *D. pulex* (Klaper *et al.*, 2009).

Chronic exposure of *H. attenuata* to nC_{60} was associated with clubbing and retraction of tentacles (EC_{50} < 10 ppm, 96 h) (Blaise *et al.,* 2008), while *D. melanogaster* exposed during the larval stages showed only slight effect at SMART (somatic mutation recombination test) on wing cells (LOEC = 2.24 ppm, NOEC = 0.45 ppm). Fullerols were not effective (NOEC = 2.46 ppm) (Zakharenko *et al*., 1997). nC60 (0.1-1 % in soil) did not affect reproduction in the potworm *Enchytraeus crypticus* (Baird, 2007), but was able to inhibit cocoon formation in *E. veneta* (Scott-Fordsmand *et al.,* 2008).

Bioavailability and bioaccumulation of nC_{60} were measured in *D. magna.* The maximum intake of carbon was greater than 2 ppm/mg of tissue after a 48 h exposure (Oberdörster *et al.*, 2006a), mainly localized in the gut. A near to complete excretion of carbon clumps organized at micrometer level was recorded after 48 h clearance, and no bioaccumulation of nanoparticles seemed to occur (Baun *et al.,* 2008). The complete clearance was accompanied by a complete recovery from toxic effects (Lovern *et al.*, 2007).

The toxicity of CNT seemed to be lower, or even absent, MW proved to be generally more aggressive than DW and SWCNT, which moderately affected the mortality rate of *D. magna* fed on algae. However, SWCNT coated with phospholipoproteins (PL-SWCNT) protected *D. magna* from mortality due to starvation. Cladocerans seemed to be able to metabolize the LP-SWCNT: when starved, they ate the lipid coating, causing aggregation and precipitation of nanotubes. Nanocarbon, mainly internalized in the gut within 45 min, was completely excreted in clumps of amorphous carbon after 20 h, or sooner if

Table 4 Summary of sublethal toxicity for fullerenes

Fullerene	Species	NOEC	DOSE	Time	Effect
nC_{60} stirred	*D. magna*	0.18 ppm	LOEC = 0.26 ppm	60 min	Swimming and feeding movements
		1.0 ppm	LOEC = 2.5 ppm	21 days	Reduced reproduction
	D. pulex	20 ppm	LOEC = 100 ppm	24h	Oxidative stress
	H. attenuata		EC_{50} < 10 ppm	96 h	Morphological changes
	E. veneta		1000 mg/kg d.w.f.		weight gain reduction (20 %) cocoon formation, not hatchability (by 78 %)
	Enchytraeus crypticus	2000 ppm		14 days	Reproduction not affected
Fullerols	*D.melanogaster*	0.45 ppm	LOEC = 2.24 ppm		SMART
		2.46 ppm			
		24 ppm			
	D.pulex	7.5 ppm	LOEC = 20 ppm	24 h	Oxidative stress
nC_{60}/nC_{60}	*D.pulex*	5.0 ppm	LOEC = 7.5 ppm	24 h	Oxidative stress
SWCNT	*H.attenuata*		EC_{50}: 10-100 ppm	96 h	
	A.tenuiremis	10 ppm		35 days	
DWCNT	*E. veneta*		EC_{50}: 94 mg/kg d.w.f.	28 days	weight gain reduction (20 %)
			EC_{10} = 37 and EC_{50} = 176 mg/kg d.w.f.	28 days	cocoon formation, not hatchability (by 60 %)

EC_{50}: Median Effective Concentration; NOEC: Non Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration; CNT: Carbon NanoTubes: SW: Single Walled, DW: Double Walled, MW: Multi Walled; SMART: somatic mutation recombination test; d.w.f.: dry weight food.

algae was provided for feeding (Roberts *et al.,* 2007).

Exposure to maximal concentrations of SWCNT did not affect mortality rates in *T. platyurus* (24 h), *H. attenuata* (96 h) and in copepod *Amphiascus tenuiremis* (28-35 days) (Templeton *et al.*, 2006; Leeuw *et al*., 2007; Blaise *et al.,* 2008). Clubbing of tentacles were present in *H. attenuata* exposed to sublethal doses (EC_{50}: 10-100 ppm, 96 h; Blaise *et al.,* 2008). *A. tenuiremis* tolerated without side effects the presence of purified SWCNT (≤10 ppm) along its entire life cycle (up to 35 days). Mortality and fertility rates, sex ratio and viability of the offspring were not affected, and a delay of 1 day in offspring development was the only side effect registered. However, the presence of fluorescent byproducts of fullerenes in the solution, possibly related to procedures adopted to obtain suspension, led to an 80 % mortality rate (Templeton *et al.*, 2006).

D. melanogaster larvae well tolerated the ingestion of SWCNT mixed with yeast feeding paste, and the mortality and fertility rates were not affected, notwithstanding the occurrence of nanocarbon accumulation in tissues and fluids (Leeuw *et al*., 2007).

DWCNT (10-30 nm diameter, 5-15 μm length) were tested on *E. veneta*. Nanotubes were mixed with food (50-495 mg/kg dry weight food, d.w.f.) and administered to worms according to standard protocols (10 g mixed food every 7th day for 28 days, ASTME1676-99, EPA850.6200; OECD211). No lethality was observed, but the exposure to the highest dose reduced weight gain (EC_{10} = 94 mg/kg d.w.f.) and cocoon formation (EC_{10} = 37 and EC_{50} = 176 mg/kg), but not hatchability.

MWCNT retained the highest toxicity, among different types of CNT: lethality rate was enhanced in *C. dubia*, *H. azteca* and *L. plumulosus* (Kennedy *et al.,* 2008, Zhu *et al.,* 2006). The only exception was *D. magna* (LC_{50} = 22.75 *vs* 2.42 ppm, MW *vs* SWCNT, 48 h). These values were obtained in a recent work, in which disagreement with previous results were commonly observed with all the materials tested (Tables 3-5): the reason, as discussed by the Authors, could be found in the different modalities for suspension preparation, and especially in continuous stirring and shaking of test medium during the exposure period (Zhu *et al.,* 2009).

Hydroxylation, carboxylation and coating with Natural Organic Matter (NOM) reduced mortality rate, at least in *C. dubia*. Survivors showed anomalies of the carapace, to which nanoparticles can adhere (Baun *et al*., 2008). Carbon accumulated mainly in the gut; it was completely excreted after 24 h washing-out and refeeding. (Kennedy *et al.,* 2008).

Table 5 Summary of lethality data collected for metal oxides organized at nanoscale

NANO-COMPOUND	TEST SPECIES AND MEDIUM	LETHALITY	REFERENCE
TiO_2:			
<20 nm	*D.magna*	48 h LC_{50} = 143 ppm	Zhu *et al.,* 2009
25-30 nm	*D magna*	48 h LC_{50} = 5.5 ppm	Lovern and Klaper, 2006
	D.pulex	48 h LC_{50}> 10 ppm	Griffitt *et al.,* 2008
	C.dubia	48 h LC_{50}> 10 ppm	
	C.elegans	24 h LC_{50} = 80 ppm	Wang *et al.,* 2009
	P.scaber	14 d NOEC >1000 ppm	Drobne *et al.,* 2009
Al_2O_3 (60 nm)	*C.elegans*	24 h LC_{50} = 82 ppm	Wang *et al.,* 2009
Ag oxide (20-30 nm)	*D.magna*	48 h LC_{50} = 0.04 ppm	Griffitt *et al.,* 2008
	D.pulex	48 h LC_{50} = 0.067 ppm	
	C.dubia		
Al oxide: 20-30 nm; or 51 nm	*D.magna*	48 h LC_{50}> 162 ppm	Zhu *et al.,* 2009
	D.pulex	48 h LC_{50}> 10 ppm	Velzeboer *et al*., 2008*;* Griffitt *et al.,* 2008
	C.dubia	48 h LC_{50} = 3.99 ppm	
Co oxide (10-20 nm)	*D.pulex*	48 h LC_{50}> 10 ppm	Griffitt *et al.,* 2008
	C.dubia	48 h LC_{50} = 1.67 ppm	
CuO:	*D.magna*	48 h LC_{50} = 3.2 ppm	Heinlaan *et al*., 2008
30 nm, or	*T. platyurus*	48 h LC_{50} = 2.1 ppm	
15-45 nm	*D.pulex*	48 h LC_{50} = 0.06 ppm	Griffitt *et al.,* 2008
	C.dubia	48 h LC_{50} = 0.419 ppm	
Ni oxide (5-20 nm)	*D.pulex*	48 h LC_{50} = .89 ppm	Griffitt *et al.,* 2008
	C.dubia	48 h LC_{50} = 0.674 ppm	
SiO_2 (205>4,700 nm)	*D.magna*	48 h, LC_{70} = 10 ppm	Adams *et al.,* 2006
ZnO:			
20 nm	*D.magna*	48 h LC_{50} = 1.5 ppm	Zhu *et al.,* 2009
50-70 nm	*D.magna*	48 h LC_{50} = 3.2 ppm	Heinlaan *et al*., 2008
	T. platyurus	48 h LC_{50} = 0.18ppm	
480>4,000 nm	*D. magna*	48 h LC_{73} = 0.2 ppm	Adams *et al.,* 2006
	C.elegans	24 h LC_{50} = 2.3 ppm	Wang *et al.,* 2009
$CuZnFe_2O_3$/Indium tin oxide/Ho_2O_3	*T. platyurus*	48h, LC_{50} = 0.1-1.0 ppm	Blaise *et al.,* 2008
	H. attenuata	96 h, EC_{50} = 10-100 ppm	
$NiZnFe_2O_3$/O_3Sm_2 / Er_2O_3	*T. platyurus*	48h, LC_{50} = 1-10 ppm	Blaise *et al.,* 2008
$SrFe_{12}O_{19}$/TiO_2/$Fe_5O_{12}Y_3$	*T. platyurus*	48h, LC_{50} = 10-100 ppm	Blaise *et al.,* 2008

Kinetic of intake and depuration rates after ingestion of MWCNT (diameter: 30-70 nm) and SWCNT (1-2 nm diameter) mixed with sediments were studied in *L. variegatus*. Data were expressed as BASFs (Biota–sediment accumulation factors, calculated as the ratio of the concentration of a substance in an organism normalized by the organism lipid fraction to its concentration in the sediment normalized by its organic carbon fraction, mg/g dry sediment (Petersen *et al.*, 2008b). At the time (7 days) of first observation, the accumulation had reached maximal levels and remained stable for the entire 28 days period of observation. Values were slightly larger for MWCNT (0.40 *vs* 0.28 mg/g dry sediment). Mortality did not increase; sub-lethal toxicity was not tested. Clean sediments added to water accelerated the clearance, nearly complete in about 2 days, and still incomplete after 60 h in clean water without sediments (Petersen *et al*., 2008b). A similar work performed in *E. foetida* gave similar results (Petersen *et al*., 2008a).

Metal oxides

These compounds behave differently from fullerenes. Particle and aggregate size are more uniformly distributed, and their hydrophobicity is generally lower, allowing easier standardization in preparing aqueous suspensions.

Table 5 shows lethality data for metal oxides. It is evident that TiO_2 is by far the most studied among this group of compounds (Fig. 2). Two freshwater invertebrates, *T. platyurus* and *H. attenuata*, permitted to group metal oxides into three toxicity degrees: $CuZnFe_2O_3$, Indium-tin oxide and Ho_2O_3 scored highest, $NiZnFe_2O_3$, O_3Sm_2, and Er_2O_3 retained an intermediate toxicity level, while TiO_2, $SrFe_{12}O_{19}$, and $Fe_5O_{12}Y_3$ scored the lowest. Moreover, compounds listed in the two last groups did not show toxic sub lethal effects on *H. attenuata* (Blaise *et al.,* 2008). When metal oxides (size range: 5-50 nm) were compared in different organisms, the toxicity degree was Ag> Cu> Ni> Co = Al = Ti in *D. pulex*, and Ag> Cu> Ni> Co> Al> Ti in *C. dubia*. Nanoparticles toxicity in comparison with that of the corresponding bulk salts gave contrasting results in different species: in aquatic organisms (*D. pulex, C. dubia*) toxicity was greater for nanoparticles, in *C. elegans* for bulk salts (Griffitt et al., 2008; Wang *et al.*, 2009). The toxicity degree of larger particles (>200 nm) was ZnO> SiO_2> TiO_2 in *D. magna*

(Adams *et al.,* 2006), partially confirmed in a recent study which found the toxicity degree to be ZnO> TiO_2> Al_2O_3 (Zhu *et al*., 2009). Highly sized (>500 nm) TiO_2, AlO_2 and CeO_2 did not retain toxicity against *C. sphaericus*: the solutions containing the higher concentrations (10 and 100 ppm) however were cloudy and unstable (Velzeboer *et al.,* 2008). Reproduction was negatively affected in *C. elegans* (Wang *et al.,* 2009). Here again, not only the chemical species, but also the modalities of suspension preparations and the size of nanoparticles were influent on toxicity.

Preparing TiO_2 suspension by filtration lead to ultra fine particulate (25-30 nm): this suspension is much more toxic than those obtained by sonication (particle size: >100 nm). Particles larger than 100 nm are poorly or nontoxic for *D. magna, T. platyurus* and *C. sphaericus* (Adams *et al.,* 2006; Heinlaan *et al.,*2008; Lovern and Klaper, 2006; Warheit et al., 2007; Blaise *et al*., 2008; Velzeboer *et al.,* 2008).

Sublethal toxicity tests were performed in two crustaceans, *D. magna*, inhabiting freshwater, and *P. scaber*, adapted to wooden soil. Filtered TiO_2 particles (30 nm) did not alter stereotypical movements of *D. magna* after 60 min exposure to the LOEC = 2.0 ppm (Lovern and Klaper, 2006; Lovern *et al.*, 2007). An immobilization test of *D. magna* after 48 h exposure to 100 % anatase TiO_2 particles (30 and 100 nm) gave unclear results: smaller and photo-catalysed particles seemed to be more toxic, but statistical difference was never reached (Hund-Rinke *et al.*, 2006).

NanoTiO_2 (10, 100 and 1,000 ppm) was not toxic when mixed with food for 14 days to *P. scaber*. Enhanced feeding rate, assimilation efficiency, levels of catalase and glutathione-S-transferase where observed: these effects appeared earlier at higher dose (3,000 ppm). Again, sonicated and bigger particles were ineffective (Jemec *et al.*, 2008; Drobne *et al.*, 2009).

Quantum dots, QDs

In addition to a number of possible interesting uses in optical and computer sciences, quantum dots represent a flexible and interesting dye for living cells and organisms. Their toxicity, bioaccumulation and clearance efficiency in invertebrate organisms have been tested in only a few species: *C. dubia*, *H. attenuata* and *Elliptio complanata*, in addition to a simplified food web including rotifers. The potential for lethality of QDs seems to be determined by their metal core, its position inside the shell and dissolution rate.

QDs with a CdSe crystalline core of 4 nm inside a shell of ZnS coated with organic polymer (total size: 15-20 nm) and registered QDs (545 ITK Carboxyl Quantum Dots) were diffusely internalized in *C. dubia*, whose body, and especially the gut, showed intense fluorescence. The toxicity was nevertheless absent (NOEC = 600 or 110 ppt, respectively; Bouldin *et al.*, 2008; Ingle *et al.,* 2008).

E. complanata was sensitive to toxic effects of QDs with CdTe (instead of CdSe) crystal core. Mussels, collected in the field and exposed for 24 h to QDs dispersed in tanks of freshwater, showed lipid peroxidation of gills and gut, reduced viability and immune activity of hemocytes (EC_{50} = 2-4 ppm; Gagné *et al*., 2008).

QDs formed by CdSe core asymmetrically sited inside a rod-shaped shell of CdS induced non-synchronous tentacle retraction, a behaviour anticipating the beginning of sexual reproduction in *H. attenuata.* The test substance was not internalized, and the dose-independent effect developed only in the presence of functioning nerves in the test organisms (intact animals or segments of their bodies). The dipole moment retained by asymmetrical QDs was probably a key requisite for inducing the anomaly: particles in which the core was symmetrically disposed inside the shell were lacking both QDs properties and toxicity (Malvindi *et al.*, 2008). Identical particles, solubilized by coating with polymers, instead were able to enter the test species: after localization in the head (within 1 h), diffusion to the entire body followed within 24 h. These nanoparticles were able to enter dissected cells of *Hydra* sp. (Tortiglione *et al*., 2007).

Finally, a simplified food web did not reveal bioaccumulation or biomagnification: *E. coli* was unable to internalize QDs, however those biotynilated or carboxylated adhered to the bacterial surface, and caused cells aggregation. Consequently, the ciliate predator (*Tetrahymena pyriformis*) avoided eating aggregates; but internalized QDs with water ingestion. Neither degradation occurred nor release of toxic metals from the core. The bioaccumulation was low and the excretion rate efficient ($T_{1/2}$ = 1.5-3.6 days). The biomagnification in the rotifer predating ciliate was very low (0.29-0.62), the $T_{1/2}$ = 14-21 h. No toxicity was found in protozoans or in predating rotifers (Holbrook *et al.*, 2008).

Other nanosized materials

A pioneering study on material possibly organized at nanoscale was carried out in 1997, using as test substance, a sonicated suspension of sucrose polyester oil, proposed as food additive for humans. Droplets were not measured. After having been exposed *in vivo* and *in vitro* to this suspension, *M. edulis* showed signs of oxidative stress, persistent lipofuscin formation and reduced lysosomes membrane stability (Moore *et al.*, 1997).

NanoPt coated with polyvinylpyrrolidone (size: 2.4 nm) acts as an antioxidant agent in larvae of *C. elegans* at L4 development stage. The mean life span was prolonged in wild-type worms and in short-living mutants *mev-1*, and the oxidative stress induced by paraquat was partially counteracted at the LOEC = 5 mM. (Kim *et al*., 2008).

M. edulis reacted to 24 h exposure to nanoAu (GNP, Gold 0 (stable), 13 nm, and 0.75 ppm) with enhanced stress parameters in digestive glands, mantle and haematocytes. Paradoxically, GNP partially protected from the oxidative stress due to menadione (Tedesco *et al*., 2008).

NanoAg (LC_{50} = 125 ppm, 24 h) accumulated in the gut of *D.magna* and on antennae in individuals near to death (Oberdörster *et al*., 2006b).

Nano^{60}Co was tested on young adults *E. foetida* maintained on standard soil. Radioactive particles obtained by neutron activation emitted

beta and gamma radiation (0.157 KBq/μg) used as a tracer, and were administered mixed with food for 7 days (667 g horse manure containing 87 μg nanoCo, or 13.7 KBq, per g). No toxicity was observed, while radioactivity was found in many tissues: spermatogenic cells, clitellum, cocoons and blood among others. A low intestinal clearance followed the depuration period (20 % within 8 weeks): the Authors considered this work a pilot study on the kinetics of nanoparticles in biological systems (Oughton *et al.*, 2008).

Upconverted phosphors (UCP) are a promising new class of dyes with application, as an example, in biomedical researches. These particles consist of trivalent ions of lanthanides embedded in a lattice of crystalline chromophores. These last act as an antenna and permit the transfer of one-two photons to the ions, otherwise unable to absorb light. Decaying from the excited state, the trivalent ions emit a luminescence characterized by its brightness and long-decay time. UCP sized 150 nm were proved non-toxic within 24 h in *C. elegans* immobilized with Na azide (NOEC = 0.5 ppm). Nanospheres were internalized in the gut, and completely excreted after 2 h only in re-fed worms (Lim *et al*., 2006).

Glass wool, used to absorb material from floating oil spill barriers, is a form of silica oxide organized in nanowires. Those used in the study (diameter: 5-25 nm, length: several microns) accumulated in lysosomes and endosomes of gills, and in mitochondria of the hepato-pancreas of *M. edulis* as shorter fibres, 100-200 nm and 60 nm, respectively. Membrane stability of lysosomes was reduced by 70 % within 24 h, and chronic exposure for 16 days enhanced lipofuscin in the hepatopancreas (Koelher *et al.*, 2008).

The commercially available polymethyl-methacrylate (PMMA, diameter: 60 nm-1.08 μm) retained toxicity independent from particle size when tested by the Chidotox test, on *Chidorus sphaericus*: (LC_{50}> 100 ppm, 48 h) (Velzeboer *et al.,* 2008).

Two organic nano-copolymer particles used in medicine (Poly *N-iso*propylacrylamide, PNIPAM and *N-iso*propylacrylamide-*co*-*N*-*tert*-butylacrylamide, NIPAM/BAM, with three different ratios of the comonomers) were tested in freshwater organisms, *D. magna* and *T. platyurus* in addition to primary consumers. The stability of suspension in water was greatly dependent from temperature, in the range 10-25 °C. NIPAM/BAM, 50:50 showed the higher toxicity, and *D.magna* was even in this case more sensitive than *T.platyurus*, (NOEC = <50 *vs* <200 ppm; LOEC = 50 *vs* 200 ppm; LC_{50} = 61 *vs* 353 ppm, respectively; Naha *et al*., 2009).

Conclusion

The importance of invertebrates to test the potential toxicity of nanomaterials can be educed from the data discussed in this paper. Invertebrate tests should not be considered as opposed to tests on other systems; instead, invertebrates add valuable information, at an intermediate level between prokaryotes and vertebrates, on pollution affecting the environment. Moreover, the wide degree of standardization, time and cost effectiveness, and suitability to study acute lethality as well as long-term toxicity represent additional advantages.

In conclusion, nanoparticles showed their elusive nature in ecotoxicological studies, leading to partially inhomogeneous results. The chemical nature of the compound, the sensitivity of the test species, the dose and the time scheduling obviously played a significant role. To them, however, the effects exerted by the nanoparticle properties should be added, so that different manipulations of pristine material and suspension generated confusion. The aggregates shape and dimension greatly influenced the results, in strict dependence from test media composition and physical-chemistry variable, pH and temperature among others. (Lovern and Klaper, 2006; Oberdörster *et al.*, 2006, Zhu *et al.*, 2006; Baun *et al.,* 2008). Functionalized surfaces, by coating, or by hydroxylation or carboxylation, generally reduced the toxicity of fullerenes (Lovern *et al.*, 2007; Kennedy *et al.,* 2008; Klaper *et al.*, 2009). Even more impressive were the results obtained by introducing apparently innocent variations, like stirring and shaking during the entire exposure time. These simple modifications were associated with unexpected changes (Zhu *et al.*, 2009).

While metal oxides seemed to retain higher ecotoxicity for invertebrates, followed by buckminsterfullerenes and MWCNT, generalization is difficult and with some degree of arbitrariness. Nanomaterial interactions with living systems are quite complicated: they can act as carriers for drugs, toxic chemicals, and dissolved substances, and enhance the bioavailability of other molecules (Zhang *et al*., 2007; Sun *et al*., 2008). Daphnid sensitivity in acute testing was high with all tested substances. A dose-dependent response to concentration or exposure time was frequent, but not always present. The concentration was indeed self-limiting, being that these compounds were poorly soluble. Chronic toxicity was a rare event, but persistent, and able to affect the population size.

In the case of QDs, generalization is difficult. The apparent low or even absent toxicity of tested formulations on invertebrates is contrasting with data from other systems (Tang *et al.*, 2008; King-Heiden *et al.*, 2009). Evidences in invertebrates, however, remain quite incomplete. Only very recent papers started a systematic study on bioavailability, bioaccumulation, compartmentalization and degradation of QDs in exposed invertebrates (Jackson *et al.*, 2009; Peyrot *et al.*, 2009).

Biodegradation and bioaccumulation were poorly studied. Both fullerene and nano-metal oxide mainly accumulated in the gut, at least in Daphnids: a nearly complete faecal excretion of digested bulk material within 20-24 h prevented bioaccumulation, as demonstrated at least in *D. magna* and *C. dubia*. Great amounts of nanoparticles (fullerene and metal oxide ot nanoAg) were instead found accumulated in all tissues of dead daphnids (Oberdörster *et al*., 2006b; Roberts *et al.,* 2007; Kennedy *et al.,* 2008). They were able to adhere to the carapace of daphnids and copepods, while the link to toxic physiological or behavioral effects was

only postulated, not documented (Baun *et al.,* 2008). Validation protocols for assessing bioaccumulations of soil pollutants in terrestrial organisms have been planned by the OECD (Egeler *et al.*, 2009).

A need for better standardization and supervision of studies seems urgent, to avoid dispersal of efforts and accumulation of anecdoctical and poorly descriptive results. Planning research under a rational of feasibility and finding easy methods is a positive trend and should be encouraged, with the limit that the comparison with different and previous studies will not be compromised, and the conventional protocols fulfilled.

Another regrettable point is the complete lack of molecular data: 48 % of about 30 genes found over- or under-expressed in human cells exposed to carbon nanotubes (Cui *et al*., 2005; Sarkar *et al*., 2007) are conserved in Bilateria and the correspondent genes are known at least in the model organisms *D. melanogaster* and *C. elegans*. Data on gene expression changes caused by exposure to nanomaterials in invertebrates should clarify the modalities of action and the underlying pathogenesis mechanisms; a wide place for new researches seems to be available in this field.

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